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Limit of Detection and Threshold for Positivity of the Centers for Disease Control and Prevention Assay for Factor VIII Inhibitors

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Abstract

Background—The Bethesda assay (BA) for measurement of factor VIII (FVIII) inhibitors called for quantitation of positive inhibitors using dilutions producing 25–75% residual activity (RA), corresponding to 0.4–2.0 Bethesda units, recommending use of “more sensitive methods” for samples with RA closer to 100%. The Nijmegen modification (NBA) changed the reagents used but not these calculations. Some specimens negative by NBA have been shown to have FVIII antibodies detectable by sensitive immunologic methods.

Objective—To examine the performance at very low inhibitor titers of the Centers for Disease Control and Prevention (CDC)-modified NBA (CDC-NBA), which includes preanalytical heat inactivation to liberate bound anti-FVIII antibodies.

Methods—Specimens with known inhibitors were tested by CDC-NBA. IgG₄ anti-FVIII antibodies were measured by fluorescence immunoassay (FLI).

Results—Diluted inhibitors showed linearity below 0.4 Nijmegen-Bethesda units (NBU). Using 4 statistical methods, the limit of detection of the CDC-NBA was determined to be 0.2 NBU. IgG₄ anti-FVIII antibodies, which correlate most strongly with functional inhibitors, were present at rates above the background rate of healthy controls in specimens with titers ≥ 0.2 NBU and showed an increase in frequency from 14.3% at 0.4 NBU to 67% at the established threshold for positivity of 0.5 NBU.

Conclusions—The CDC-NBA can detect inhibitors down to 0.2 NBU. The FLI, which is more sensitive, demonstrates anti-FVIII IgG₄ in some patients with negative (<0.5) NBU. The sharp

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¹See Appendix for list of the Hemophilia Inhibitor Research Study Investigators

Addendum

C. H. Miller analyzed results and wrote the manuscript. B. Boylan performed the research, analyzed the results, and wrote the manuscript. A.D. Shapiro, S.R. Lentz, and B.M. Wicklund provided patient samples and critically revised the manuscript. The Hemophilia Inhibitor Research Study Investigators provided patient samples and data and reviewed the manuscript.

increase in IgG₄ frequency between 0.4–0.5 NBU validates the established threshold for positivity of 0.5 NBU for the CDC-NBA, supporting the need for method-specific thresholds.

Keywords

Factor VIII; inhibitor; hemophilia

Introduction

The “gold standard” assay for measurement of factor VIII (FVIII) inhibitors is the Nijmegen modification [1] of the Bethesda assay (BA) [2], according to International Society on Thrombosis and Haemostasis guidelines [3]. The Nijmegen-Bethesda assay (NBA) modified the reagents used in the BA but maintained its method of calculation of the inhibitor units based on a ratio of the FVIII activity in a mixture of patient plasma and normal pooled plasma (NPP) to the FVIII activity in a control mixture, expressed as the % residual activity (RA). The original description of the BA in 1975 [2] recommended that quantitation of positive inhibitors be carried out using %RA between 25% and 75%, corresponding to 0.4–2.0 Bethesda units (BU), and, more recently, it has been suggested that the limit of detection of the BA is 0.4 NBU [4]. Although the graph provided by Kasper et al. (2) showed linearity to 100% RA, the authors recommended use of “more sensitive methods” for RA close to 100%, an assertion that is supported by several more recent studies using sensitive immunologic methods to detect FVIII antibodies in specimens found to be negative by BA or NBA [5–10]. FVIII activity measurement methods have changed over the past 40 years, and documentation is lacking describing the performance of the BA and NBA at low titers using today’s automated methods to assign FVIII activity levels. The ability to accurately measure inhibitors in the 0–0.4 NBU range would allow for more precise monitoring of developing inhibitors. To this end, we have previously validated modifications to the NBA to free anti-FVIII antibodies bound to FVIII and increase its precision [12] in order to minimize the number of false negative results, and assay modifications to increase the sensitivity of the NBA have been proposed for use in monitoring of immune tolerance induction therapy (ITI) [11]. In this report, we examined the performance of the CDC-modified Nijmegen-Bethesda assay (CDC-NBA) at very low inhibitor titers in a single laboratory and used measurement of anti-FVIII antibodies to validate the limit of detection and threshold for positivity established.

Materials and Methods

Specimens for FVIII inhibitor testing were collected from subjects with congenital hemophilia A enrolled in the Hemophilia Inhibitor Research Study between 2006–2012 at 17 U.S. Hemophilia Treatment Centers, as described in detail elsewhere [12, 13]. Healthy control subjects were paid donors with no history of coagulation disorder.

FVIII inhibitor measurements were performed by the CDC-NBA, as previously described [12]. Specimens were tested undiluted and at serial two-fold dilutions of 0.5, 0.25, 0.125, etc., in FVIII-deficient plasma (FVIIIIDP, George King Biomedical, Overland Park, KS) until a dilution with 100% RA was reached. Antibodies of IgG₄ subclass binding to FVIII were

measured by fluorescence immunoassay (FLI) as previously described [9], in 382 specimens. The threshold for positivity for IgG₄ antibodies was set at 2 standard deviations above the mean for 56 healthy subjects [9].

Dilution curves were evaluated by linear regression with significance of deviation from 0 slope calculated by F test and linearity by runs test with a significance level of $P < 0.5$. Limit of detection (LOD) of the assay was estimated by four methods. Method 1: using the classical method as described by the Clinical and Laboratory Standards Institute (CLSI) [14], 4 blank specimens prepared from equal parts normal pooled plasma (NPP) and FVIIIIDP and 4 low level specimens of 1.0 NBU prepared by diluting high-titer inhibitor plasma (George King) in imidazole buffer (Siemens, Marburg, Germany) were tested using 2 different lots of assay reagents over 3 days with a total of 60 replicates of each type of specimen. Non-parametric methods were used to calculate limit of the blank (LOB) as the 95th percentile rank for negative specimens. LOD was calculated as the LOB plus the standard deviation (SD) of the low level specimens corrected to the 95th percentile of the normal distribution. Method 2: the lowest dilution detected, i.e., having less than 100% RA (> 0 NBU), was calculated for 17 FVIII inhibitors of 1.0–24.6 NBU. Method 3: linear regression of the lower dilutions of 14 positive inhibitors with at least 3 dilutions between 50% and 100% RA: 1.1–1.7 NBU ($n=5$), 6.7–9.6 NBU ($n=4$), 13.1–14.9 NBU ($n=3$), and 23.0–24.6 NBU ($n=2$), was performed. Mean and SD of the NBU at which the regression line reached 100% RA and slope were calculated, and the LOD was calculated as $3SD/\text{slope}$. Method 4: specimens from 30 healthy subjects were tested and LOD was calculated as $\text{mean} + 3SD$. Analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA).

Results and Discussion

The CDC-NBA has been previously shown to have a coefficient of variation (CV) between runs for the negative control of 9.8% ($n = 117$) and for the 1.0 NBU positive control of 10.3% ($n=114$). Within-runs variation showed a CV of the negative control of 5.3% ($n = 10$) and of the positive control 4.8% ($n = 10$) [12]. The CDC-NBA was performed on serial dilutions of high (13.1–24.6 Nijmegen-Bethesda units (NBU); Figure 1A), low (1.1–1.7 NBU; Figure 1B), and very low/negative (0.6 and 0.8 NBU/ <0.5 NBU; Figure 1C) specimens in order to assess the assay's ability to measure inhibitors in specimens containing inhibitors approaching 100% RA. Linearity of each curve was verified by a runs test. All positive specimens showed linearity with a significant negative slope. Linearity between 100% and 75% RA suggests that NBU can be read below 0.4 NBU; however, the curves did not cross the y-axis at 100% RA, indicating that the LOD of this assay is not 0 NBU. As shown in Figure 1C, very low positive specimens (0.6 and 0.8 NBU) showed a similar change with dilution, while negative specimens showed a consistent %RA when diluted and lacked a negative slope. Observation of multiple dilutions, even when not required by the assay protocol, may be useful in distinguishing very low titer inhibitors from negative specimens.

Calculation of LOD, the lowest level at which inhibitor can be detected, by 4 standard methods is shown in the Table. All four statistical methods used indicate that the lower limit

of detection of the CDC-NBA, which is reported to one decimal place, is 0.2 NBU. Similar calculations can be carried out by clinical laboratories using specimens prepared from assayed plasmas obtained commercially or from laboratories using validated methods.

Dardikh et al developed a modified NBA which utilizes concentrated plasma in a chromogenic assay with a reported sensitivity of 0.03 NBU and reported that it detects low titer inhibitors that remain after ITI and are responsible for reduced FVIII half-life [11]. Such an assay may be superior to the NBA for this purpose; however, further studies using the FLI, which has a similar sensitivity [16], for ITI monitoring are warranted.

A threshold for positivity of 0.5 NBU for the CDC-NBA was previously established based on patient history in 674 subjects [12]; its range can be estimated based on the CV of the CDC-NBA to be 0.45–0.55. Because functional inhibitor titers have been shown to correlate most strongly with levels of anti-FVIII antibodies of IgG₄ subclass [8, 9, 17–19], we measured anti-FVIII IgG₄ in specimens at various inhibitor titers (Figure 2). Among 64 specimens with ≥ 2.0 NBU, 62 (97%) demonstrated the presence of anti-FVIII IgG₄. Those with titers of 0.6–1.9 NBU had frequencies of 79–83%. At the CDC-NBA cutoff for positivity of 0.5 NBU, IgG₄ frequency was 66.7%, decreasing to 14.3% at 0.4 NBU. IgG₄ antibodies were present in specimens with titers ≥ 0.2 NBU at rates above that seen in healthy controls [9]. These data confirm that a majority of specimens become antibody-positive at 0.5 NBU. Figure 2 also illustrates that specimens with 0.5–1.9 NBU have a lower frequency of IgG₄ positivity than higher titer specimens, confirming the false positive rate in that range previously reported with assays detecting both IgG and IgM antibodies [16]. IgG₄ anti-FVIII antibodies were absent in 19% of specimens with 0.5–1.9 NBU.

The presence of IgG₄ anti-FVIII antibodies in some specimens below the positive threshold confirms previous observations [5–10] that some patients with a negative NBA have developed an antibody response to FVIII. In some cases, these antibodies appear to have clinical effects [7, 10]. A 9-year-old HA patient with titers of 0.4 NBU and 0.4 chromogenic Bethesda units and strongly positive anti-FVIII antibodies was observed to have FVIII recovery of 10% of expected and half-life of 2 hours post infusion. ITI therapy increased his recovery to 80% and his half-life to 6 hours [unpublished observation]. These observations and the finding that patients with titers of 0.5–1.9 NBU and those with higher titers have similar levels of anti-FVIII antibodies by FLI [20] suggest that *in vitro* measurement of inhibition may not always reflect the antibody load. *In vitro* conditions may not accurately mimic those present *in vivo*, where antibody affinity may play a role in determining the effect [21]. Diagnosis of an inhibitor in a hemophilia patient is a clinical judgement and should not be included or excluded solely on the basis of a threshold for positivity.

These results document that the threshold of positivity with the CDC-NBA is slightly different from previous recommendations [3] based on the NBA without preanalytical heat inactivation, suggesting the need to re-evaluate the threshold for positivity when significant changes are made to the method. The FLI or other immunologic test may be useful as an objective measure for setting the threshold for a particular method. It has not been standard practice for each laboratory to set a reference range for FVIII inhibitors, and most laboratories lack sufficient specimens to do so. In order to use published definitions,

however, published methods must be carefully followed with any changes in procedure or substitution of reagents validated.

The CDC-NBA produces acceptable intra-laboratory variability using the positive and negative controls recommended, which can be prepared from commercially-available reagents [12]. Inter-laboratory variability was not examined in this study. Inter-laboratory variability of FVIII inhibitor results in external quality assessment programs is large, with CVs approaching 50% on distributed specimens and false-positive rates up to 32% [4, 22, 23]. This has been attributed to differences in laboratory methods and reagents. A study with 13 participants showed that a mean CV of 44% for 6 specimens using their original methods could be reduced to 8% by use of a standardized Nijmegen method and uniform reagents [23], suggesting that adoption of a standard method on a national or international level and use of validated reagents could lead to improvement. Standardization has been limited by the lack of commercial availability of key reagents, such as imidazole-buffered NPP and controls, cost of FVIII-deficient plasma, and lack of an international standard.

Introduction of new longer-acting treatment products has resulted in factor measurement issues, with differences noted based on the specific assay reagents used [24]. Inhibitor assays using preanalytical heat inactivation avoid this issue, because the calculated NBU is based on a ratio of two FVIII activity readings measuring the FVIII activity of the added NPP and not patient or infused FVIII, which has been removed. If the two FVIII measurements used to calculate an inhibitor are then carried out with appropriate quality control using calibrators allowing reporting in international units, theoretically, differences in factor assay reagents and instruments should not affect the final NBU. This, however, remains to be demonstrated. Accuracy of the test will be dependent upon the ability of the preanalytical heat inactivation step to remove any treatment product that may influence the results of the FVIII assays. This remains to be established with most significantly modified treatment products.

In conclusion, the CDC-NBA can detect inhibitors down to 0.2 NBU. The FLI, which is more sensitive, demonstrates anti-FVIII IgG₄ in some patients with negative (<0.5) NBU. The sharp increase in IgG₄ frequency between 0.4–0.5 NBU validates the established threshold for positivity of 0.5 NBU for the CDC-NBA. Further evaluation of the CDC-NBA in a multi-laboratory study is warranted.

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Appendix

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Essentials

- Immunologic methods detect factor VIII (FVIII) antibodies in some inhibitor-negative specimens.
- Specimens were tested by modified Nijmegen-Bethesda assay (NBA) and fluorescence immunoassay.
- The NBA with preanalytical heat inactivation detects FVIII inhibitors down to 0.2 NBU.
- IgG₄ frequency validates the established threshold for positivity of 0.5 NBU for this NBA.

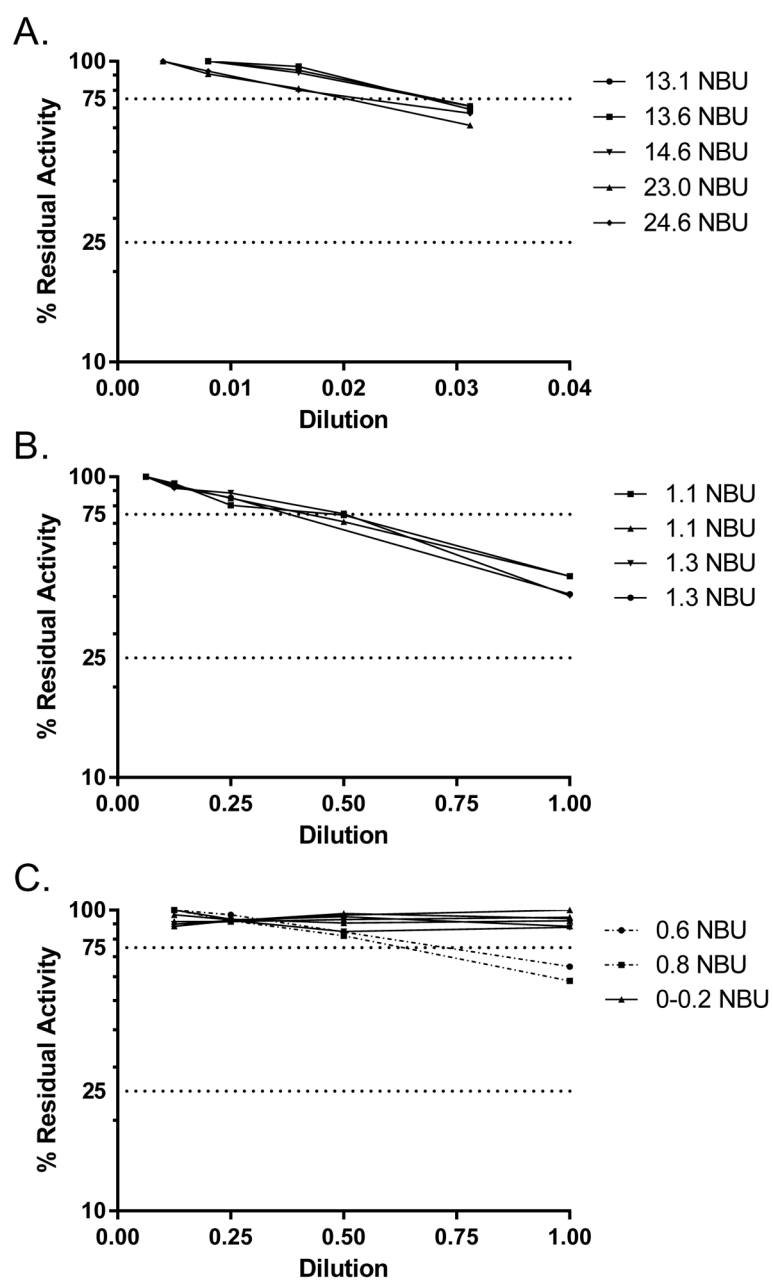


Figure 1. Dilution curves of factor VIII inhibitors measured by the CDC Nijmegen-Bethesda assay. A. Inhibitors of 13.1–24.6 Nijmegen-Bethesda units (NBU). B. Inhibitors of 1.1–1.3 NBU. C. Positive inhibitors of 0.6 and 0.8 NBU and negative inhibitors (<0.5 NBU).

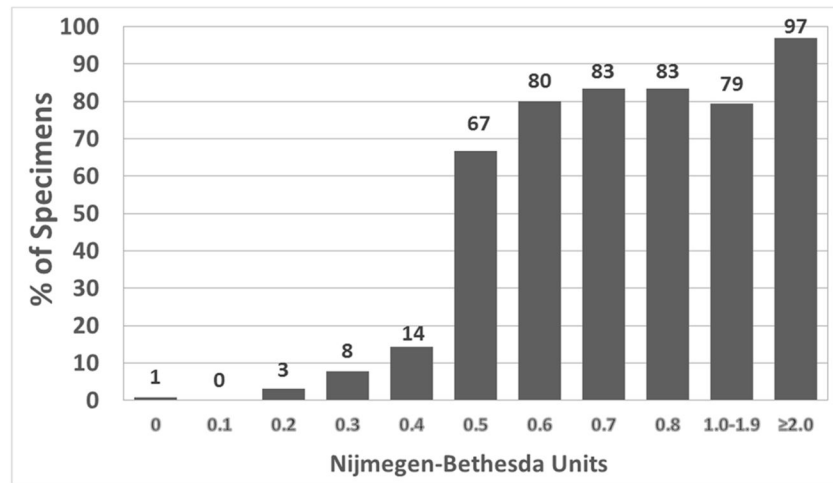


Figure 2. Per cent (%) of hemophilia A specimens positive for anti-factor VIII IgG₄ antibodies at various levels of inhibitor measured in Nijmegen-Bethesda units by the CDC Nijmegen-Bethesda assay

Table

Calculation of the limit of detection (LOD) of the CDC-modified Nijmegen-Bethesda method for factor VIII inhibitors in Nijmegen-Bethesda units (NBU)

METHOD	SAMPLES	MEAN (RANGE)	LOD NBU
CLASSICAL METHOD (CLSI) ¹⁴	4 blank, 4 low	--	0.18
LOWEST DILUTION DETECTED ¹⁵	17 (1–24 NBU)	0.19 (0.14–0.25)	0.19
LINEAR REGRESSION ¹⁵	14 (1–24 NBU)	0.06 (0.01–0.14)	0.24
NEGATIVE SPECIMENS ¹⁵	30 healthy subjects	0.03 (0–0.15) SD 0.047	0.17